CONTRIBUTIONS TO THE BIOLOGY OF RHIZOBIA.

I. RHIZOBIUM MUTABILE IN ARTIFICIAL CULTURE MEDIA.

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(WITH PLATE I)

RECENTLY I began some further work on the biology of rhizobia. The investigations carried on at the Illinois Experiment Station at Champaign in 1893 were terminated before any satisfactory results were reached, and it is only recently that an opportunity has again presented itself to further pursue the investigations.

The review of recent work done in the study of rhizobia will be given in the second paper. A fairly complete list of references up to and including the year 1897 will be found in the *Minnesota Botanical Studies* of September 27, 1894, to which a supplement was added in the *Studies* of May 29, 1897.

Since 1886 numerous investigators, especially those of Germany and France, have attempted to make pure cultures of rhizobia, and some of these have stated repeatedly that cultures were obtained, but not a single investigator has thus far given detailed information regarding them. Other investigators, again, declared that rhizobia were incapable of being grown in artificial media; that they were, indeed, merely plasmic by-products, to which the name *Bakteroiden* was given by Frank and other German investigators. Another source of confusion and difficulty was the fact that most investigators recognized only one form (or species) of rhizobium, namely, the *Rhizobium leguminosarum* of Frank. Without entering again into a discussion of the probable number of species, I shall outline briefly the present preliminary investigations.

I have made repeated attempts to obtain pure cultures of the rhizobia found in the root tubercles of *Melilotus alba*, but with either wholly negative results or only a very evanescent partial success. So frequent were my total failures that I became

almost converted to the opinion that this particular rhizobium was either not an autonomous organism, or that it had lost the ability to grow outside of the host-plant. I have had no difficulty in securing cultures of the rhizobium found in bean-root tubercles, for example, but for reasons which will be given in a subsequent paper I was desirous of obtaining cultures of the comparatively large rod-shaped to Y-shaped form found in sweet clover. For some time after beginning the present series of experiments it appeared that my efforts would again be without positive results; but my former experiences induced me to proceed more cautiously and more carefully, keeping in mind the probable physiological peculiarities of this particular rhizobium as compared with bacteria in general. Now I feel certain that in 1893 I had several small pure cultures of this particular rhizobium, but failed to recognize them, and they were destroyed without being investigated, and, unfortunately, I was furthermore obliged to discontinue the investigations at a time when I was becoming somewhat familiar with my subject.

In the following experiments young seed-grown plants of *Melilotus alba* were selected, which were about four inches high, each plant having from six to fourteen fairly well developed, mostly single, non-branching, more or less irregularly oval to flattened tubercles. The roots and tubercles were thoroughly and repeatedly washed in hydrant water, and dried with blotting paper. A tubercle was cut into with a small sterilized knife, and the knife-blade was lifted, thus breaking the tubercle across. This prevented the blade from dragging exterior bacteria over the interior of the tubercle. A short platinum needle was pushed into the central portion of the tubercle and streak (line) cultures made in Petri dishes with *Melilotus alba* extract solidified by means of agar. The inoculated Petri dishes were kept in the dark at the spring and early summer temperature of the laboratory. Most of the dishes showed white growths in a day or two. Examination proved these to consist of several motile bacteria, including a large motile bacillus resembling *B. anthracis* or *B. subtilis*. In the course of about five days from the time of

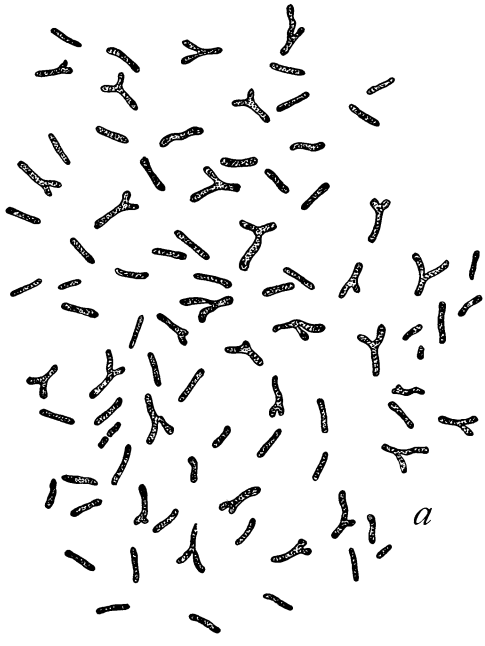
making the inoculations I noticed in several of the streaks a very slight, translucent, glistening growth, which at once reminded me of similar growths observed in my former investigations. Upon microscopic examination I found numerous organisms, some simple and irregular in form, some Y-shaped, some twice and three times forked, resembling the rhizobium of the sweet clover to which I gave the name *Rhizobium mutabile* in 1892. Of these few small growths I made a number of test-tube cultures. These cultures developed very slowly, but steadily, producing (upon agar media) a transparent, glistening, slightly elevated, smooth-margined growth of about the tenacity (not stringy) and consistency of syrup. There was no color, odor, or gas formation noticeable. Repeated microscopic examinations showed these cultures to consist of organisms having the morphological characteristics of *R. mutabile* of sweet clover. These morphological characteristics, combined with the very peculiar development of the cultures, led me to assume that I had at last succeeded in obtaining pure cultures of *R. mutabile*. H. B. Carey, working in the same laboratory, succeeded in obtaining numerous similar cultures from *M. alba* tubercles by the fractional culture method. The evidence, though requiring further proof, is sufficient to justify this report. Inoculation experiments on *M. alba* growing in carefully sterilized soil are now in progress, which will be reported upon in the second paper.

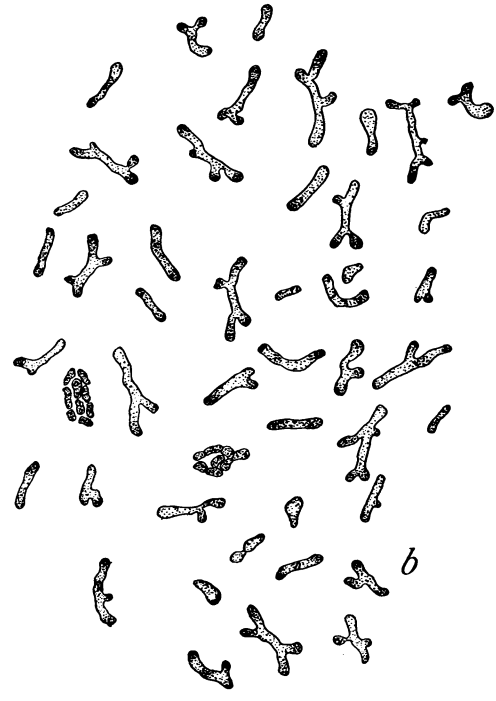
Microscopic examination of pure cultures showed the rhizobia in various stages of septation, budding, and branching. The organisms are absolutely non-motile, showing more irregularity in form, size, and branching than in their normal state within the tubercle. They are embedded in a considerable quantity of mucilaginous substance, which does not, however, form a distinctly circumscribed layer about the cell wall. Naturally the study of the mode of multiplication is a comparatively easy task with pure cultures. It multiplies by bi-septation, multi-septation, and by budding and subsequent septation. In comparatively rare instances there is evidence of budding resembling that of

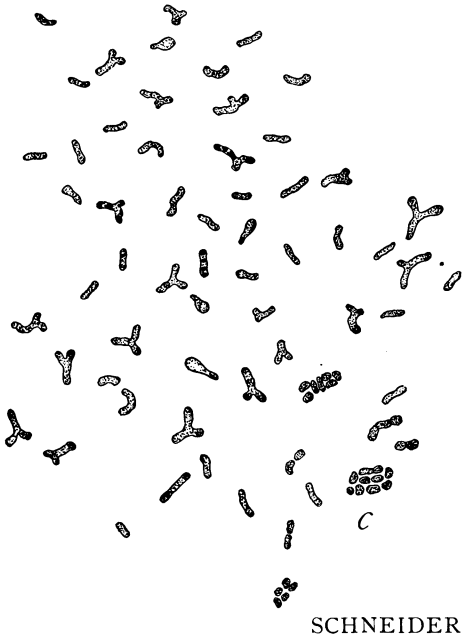
the yeast plant. Growth is usually uniterminal; again it may proceed in either direction or toward all of the extremities of the branching forms, until the maximum size is reached, whereupon it septates. Under favorable conditions, when food supply is adequate, septation proceeds more rapidly and before the maximum size is attained, forming zoöglœa of only partially developed rhizobia. When a fully matured organism septates, the cytoplasm collects into two, more generally three to five, masses, followed by a constriction of the cell-wall between the plasmic masses. Branching forms seem to be due to arrested, prolonged, or incomplete budding, and occur principally in the older, more mature plants, though it may also be noticeable in immature or undeveloped organisms. These peculiarities of form and budding indicate a relationship to Saccharomyces, but there are absolutely no fermentation phenomena in the media.

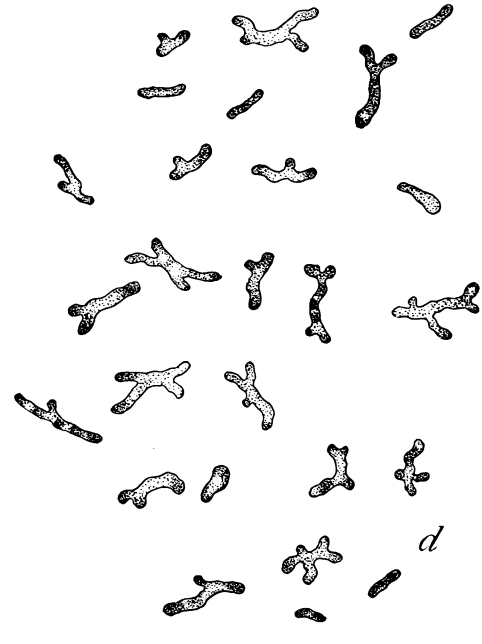
In the older, larger, and more matured cells are found those small, usually spheroidal, highly refractive bodies to which attention has been called by various investigators, notably Frank. In the culture organisms, as well as in those found in tubercles, these bodies, which vary in number from one to five, usually occupy a peripheral position. They are highly refractive, in that respect resembling spores. In 1894 I described them as modified spores, an opinion not seconded by Frank (a communication in writing). At present I have nothing to add regarding their origin and probable function, though I am still of the opinion that they may be spore-like etiologically, and might be designated sporoids. It seems evident that no true spores are formed, either in the organisms of the tubercles or those grown in media.

Rhizobium mutabile grows in most media, liquid beef broth gelatine, agar, beef broth, whether neutral, slightly acid or slightly alkaline. It seems to grow better upon solid than in liquid media. It grows best, as far as observations have been made, upon solid *M. alba* extract agar. It is not anaerobic, though it may be facultatively so. The fact that the culture is colorless renders the study of deep stab growths difficult,









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especially if the media are not wholly translucent or transparent. In liquid media there is a whitish sediment of rhizobia. There is evidence that growths on solid media become less translucent with age.

The following is a brief summary of results of the present series of experiments:

1. *Rhizobium mutabile* can be isolated and develops in artificial culture media, forming a translucent, colorless, glistening, semi-liquid growth.

2. It develops slowly, requiring from four to six days to produce a perceptible growth.

3. It is essentially aerobic.

4. It multiplies by biseptation, multiseptation, and a modified form of budding.

5. It apparently does not develop true spores.

6. It is devoid of all active motion.

7. It undergoes great changes of form and size, with age and also in different culture media. See figures.

8. Refractive bodies (sporoids) occur in the older organisms, and seem to be characteristic of the species. Their origin and function are as yet undetermined.

9. While of slow growth, it does not seem to be readily destroyed. It may be completely overspread by a foreign germ, but as soon as conditions become unfavorable for the latter the rhizobium is found to be still active and continues to grow and multiply.

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EXPLANATION OF PLATE I.

The illustrations are from pen and ink drawings, made on a uniform scale by means of an Abbé camera lucida. The drawing paper was placed on a level with the base of a Leitz instrument. Magnification by means of a B. and L. 1-12 oil immersion objective and a No. 4 Leitz ocular, draw-tube in.

a, *Rhizobium mutabile* from young root tubercle of *Melilotus alba*.

b, *R. mutabile* grown in agar beef broth.

c, *R. mutabile* grown in beef broth.

d, *R. mutabile* grown in *Melilotus alba* extract solidified by agar.